

Research Articles

Improving vector-borne pathogen surveillance: A laboratory-based study exploring the potential to detect dengue virus and malaria parasites in mosquito saliva

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ABSTRACT

Background & objectives: Vector-borne pathogen surveillance programmes typically rely on the collection of large numbers of potential vectors followed by screening protocols focused on detecting pathogens in the arthropods. These processes are laborious, time consuming, expensive, and require screening of large numbers of samples. To streamline the surveillance process, increase sample throughput, and improve cost-effectiveness, a method to detect dengue virus and malaria parasites (*Plasmodium falciparum*) by leveraging the sugar-feeding behaviour of mosquitoes and their habit of expectorating infectious agents in their saliva during feeding was investigated in this study.

Methods: Dengue virus 2 (DENV-2) infected female *Aedes aegypti* mosquitoes and *P. falciparum* infected female *Anopheles stephensi* mosquitoes were allowed to feed on honey coated Flinders Technical Associates —FTA® cards dyed with blue food colouring. The feeding resulted in deposition of saliva containing either DENV-2 particles or *P. falciparum* sporozoites onto the FTA card. Nucleic acid was extracted from each card and the appropriate real-time PCR (qPCR) assay was run to detect the pathogen of interest.

Results: As little as one plaque forming unit (PFU) of DENV-2 and as few as 60 *P. falciparum* parasites deposited on FTA cards from infected mosquitoes were detected *via* qPCR. Hence, their use to collect mosquito saliva for pathogen detection is a relevant technique for vector surveillance.

Interpretation & conclusion: This study provides laboratory confirmation that FTA cards can be used to capture and stabilize expectorated DENV-2 particles and *P. falciparum* sporozoites from infectious, sugar-feeding mosquitoes in very low numbers. Thus, the FTA card-based mosquito saliva capture method offers promise to overcome current limitations and revolutionize traditional mosquito-based pathogen surveillance programmes. Field testing and further method development are required to optimize this strategy.

Key words *Aedes aegypti*; *Anopheles stephensi*; dengue virus; FTA card; mosquito saliva; *Plasmodium falciparum*; vector-borne disease; vector pathogen surveillance

INTRODUCTION

Arthropods such as mosquitoes, fleas, ticks, and sandflies carry and transmit a large number of infectious agents, many of which cause diseases with debilitating symptoms and even death. Three such diseases that cause highest disease burden across the world are dengue fever, malaria, and leishmaniasis. The World Health Organization (WHO) estimates that each year there are approximately 250–500 million new cases of dengue, 210 million new cases of malaria, and 900,000–1.3 million new cases

of leishmaniasis¹. With high incidence of these three diseases as well as increased global travel and commerce, the distribution of various vector-borne diseases is changing at an unprecedented rate. For example, the recent emergence of Zika virus (ZIKAV) in the Americas and chikungunya virus (CHIKV) in the Caribbean are particularly alarming^{2–3}. In these instances, vector surveillance can detect the presence of a pathogen, identify transmission foci, and design appropriate intervention strategies prior to onset of human illness. Surveillance data are equally important following intervention activities. However,

current vector-borne pathogen surveillance methods are often prohibitively costly and labour intensive to implement in situations where they are most required.

Traditional arthropod-based surveillance methods rely on capturing targeted species followed by laborious steps to identify, process, and test the samples. In most arbovirus surveillance programmes, a cold chain must be maintained, further complicating the logistics. Additionally, mosquito collection methods vary and are traditionally very labor intensive. A classic review on this topic was published by Service⁴ in 1977 and methods have remained largely unchanged since then, with the exception of the advent of new traps such as the BG Sentinel and the development of new attractants. While some mosquito species are efficiently sampled with standard adult traps, this is not the case for either *Ae. aegypti* or the primary anopheline vectors of malaria. Consequently, surveillance programmes for these species are commonly limited by low trap catches, and require the use of large numbers of traps.

Following vector collection, samples must be transported to a central facility for processing and analysis. This requires manual sorting of specimens into pools, grouped by species and collection location while maintaining a cold chain and maceration for pathogen testing. Detection methods may include rapid antigen detection using lateral flow immunochromatographic assays (wicking or dipstick assays), nucleic acid detection using PCR, qPCR, or LAMP assays, or infectious virus detection using cell culture techniques followed by a plaque assay⁵. Vector infection rates are often below 1/1000 requiring very large sample sizes to accurately surveil the population, which equates to very lengthy and costly analyses.

In addition to the difficulties in vector collection and processing challenges, another surveillance complexity is that most pathogens require anywhere from several days to several weeks to replicate and disseminate to the salivary glands before they can be transmitted⁶. Because of this phenomenon, screening pools of macerated specimens for pathogen-specific antigens or nucleic acid sequences can indicate whether samples are infected (carriers), but not necessarily infectious (transmitting). The separation of these two pathologies (infected *vs* infectious) is critical to surveillance programmes and is key to identifying true vectors and estimating public health risk. Pathogen detection directly from arthropod saliva samples averts the risk of misidentifying the vectors that are merely infected with the ones that are actually infectious.

It is known that all mosquitoes salivate when they sugar feed in much the same way as when they blood-feed. This means that a portion of the dengue virus load

or malaria parasites in their salivary glands will be expectorated during feeding⁷⁻⁹. Saliva can be collected using Flinders Technical Associates—FTA[®] cards (Whatman, Piscataway, New Jersey), which are filter paper cards impregnated with proprietary chemicals designed to bind and preserve nucleic acids at room temperature¹⁰. Once the nucleic acid is extracted from the saliva on the card, PCR can be run on this nucleic acid to detect the pathogen gene of interest. A positive card indicates that the arthropods are not just infected but also capable of transmitting the pathogen of interest. This method of sample collection may allow for more accurate and cost-effective assessment of the actual risk of contracting a vector-borne disease in a given area.

Recently, the utility of FTA[®] cards has been leveraged in several studies for mosquito-borne virus surveillance, including but not limited to West Nile virus (WNV), Ross River virus (RRV), Barmah Forest virus (BFV), Japanese encephalitis virus (JEV), and CHIKV¹¹⁻¹⁴. However, this is the first report to quantitatively assess the feasibility to capture saliva and detect dengue virus and malaria parasites using FTA cards. This study discusses the potential to leverage this method to improve upon current vector-borne pathogen surveillance programmes, specifically with the growing interest in exploiting sugar feeding behaviour to control mosquitoes.

MATERIAL & METHODS

Mosquito infection

Aedes aegypti mosquitoes: A total of 200 female *Ae. aegypti* mosquitoes reared in the insectary at the Walter Reed Army Institute of Research (WRAIR) in Silver Spring, Maryland were infected with dengue virus 2 (DENV-2) (strain New Guinea C) *via* intrathoracic inoculation and were allowed to incubate for 12–14 days at 28°C with a 12 h of light, 12 h of dark cycle. In total, 160 mosquitoes were recovered (16% mortality rate), and infection was confirmed in a random 5% of this population *via* the DENV-2 reverse transcriptase (RT)-qPCR detection method described in a separate section. All mosquitoes were then housed individually for FTA card testing.

Anopheles stephensi mosquitoes: A total of 200 female *An. stephensi* mosquitoes reared in the WRAIR insectary were infected with *P. falciparum* NF54 strain parasites using a standard membrane-feeding technique. After 12 days of incubation, 182 mosquitoes were recovered (9% mortality rate), and infection was confirmed in a random 5% of the population by dissecting salivary

glands and isolating sporozoites in 200 μ l of 1x phosphate buffered saline (PBS) + 1% bovine serum albumin (BSA) with silicone coated tips and tubes (Costar[®], Rockville, Maryland) to prevent clumping and sticking of sporozoites. The sporozoite count was performed using a glass haemocytometer. Additional confirmation was done *via* the *P. falciparum* qPCR detection method as described below. All mosquitoes were then housed individually for FTA card testing.

Preparation of FTA cards

Active Manuka honey (Nelson active Manuka honey, Australia) was chosen as the source of sugar due to its high antibacterial properties. It was mixed with blue food colouring dye so that the dye could be visualized when internalized by the mosquitoes. This honey was then applied evenly on Classic or Clonesaver FTA[®] (Whatman, Piscataway, New Jersey) cards and covered by parafilm¹¹.

Mosquito survival test

Individually housed mosquitoes were split into two groups, those with a water source ($n = 12$) and those without ($n = 12$). Each group was allowed to sugar feed on blue dye and honey soaked FTA cards for 6–72 h at 27°C and 76% room humidity. At the following time-points, life status and sugar feeding was assessed: 2, 4, 6, 12, 24, 48, and 72 h. Sugar feeding was determined by the visualization of blue dye in mosquito gut or crop, and/or the presence of dyed excrement.

FTA card storage test

To determine the effects of storage conditions on the ability of FTA cards to preserve nucleic acids, 1 μ l of virus stock containing 20 plaque forming units (PFU) of DENV-2 was spotted on honey-coated 1 cm² Clonesaver FTA[®] cards (Whatman, Piscataway, New Jersey) in three sets of six. FTA cards from the first and second sets were each placed next to a molecular biology grade water (Fisher Scientific, Waltham, Massachusetts) soaked cotton ball and incubated for up to 72 h at 26°C and 70% humidity. After incubation, the first set of cards was frozen overnight at -20°C before processing, and the second set of FTA cards was placed into microcentrifuge tubes containing desiccant beads (Whatman, Piscataway, New Jersey) and stored at 4°C overnight before processing. The third set of FTA cards was allowed to dry at room temperature after addition of virus and processed after 72 h.

Mosquito FTA card sugar feeding

Two sets of 30 *Ae. aegypti* mosquitoes were injected with 12 PFU of DENV-2 viral stock. At 12 and 14 days

post-infection (pi), 30 female *Ae. aegypti* mosquitoes were sugar starved for 12 h then housed individually (Fig. 1a) in plastic vials sealed with black mesh to allow access to honey-coated Classic FTA[®] cards and water soaked cotton balls placed on top of the vials. For each set, blue food dye was added to the honey to serve as an indicator of mosquito feeding on the FTA card. Sugar feeding was permitted for 6 h and was determined by the visualization of blue dye in mosquito gut or crop, and/or the presence of dyed excrement (Fig. 1b). The viral RNA was extracted from both the mosquito and associated FTA card. FTA cards were analyzed for the presence of DENV-2 RNA by RT-qPCR. Negative extraction controls were used to assess cross-contamination during extraction.

Three sets of 20 female *An. stephensi* mosquitoes infected *via* membrane-feeding with *P. falciparum* NF54 were sugar starved for 12 h, then housed individually and allowed to feed for either 6, 24, or 65 h on the honey and dye coated Classic FTA[®] card. After feeding, the mosquitoes

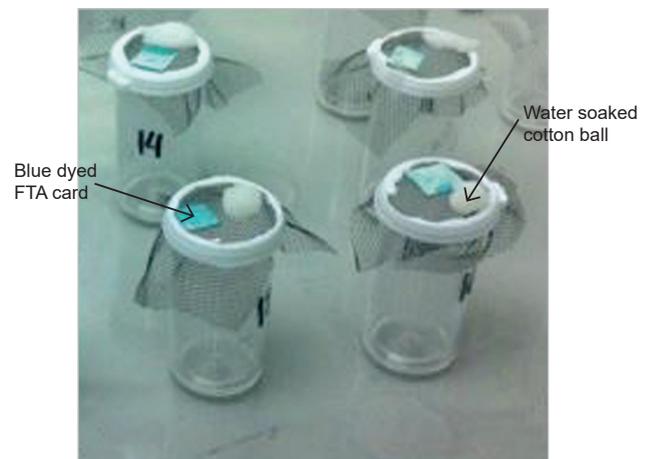


Fig. 1a: Experimental set up for both *Ae. aegypti* and *An. stephensi* FTA card sugar-feeding. Mosquitoes housed individually were provided with a 1 cm² blue-dyed, honey-coated Clonesaver FTA[®] card and a water soaked cotton ball.



Fig. 1b: *Anopheles stephensi* with blue-dyed gut after sugar-feeding on FTA card.

were dissected to visualize the presence or absence of blue dye in their midgut or crop. If present, the salivary glands were dissected from each mosquito for DNA extraction. The FTA cards were analyzed by qPCR for the presence of sporozoites excreted by mosquitoes during sugar feeding and/or probing. Negative extraction controls were used to assess cross-contamination during extraction.

Nucleic acid extraction

DENV-2 RNA was extracted from FTA cards and *Ae. aegypti* mosquitoes using the Qiagen QIAamp Viral RNA extraction kit according to the manufacturer's protocol with the additional step of manually homogenizing mosquitoes in lysis buffer AVL (Qiagen, Valencia, California) with a plastic microtube pestle. All RNA was eluted in 60 µl AVE buffer and stored at -80°C or used immediately.

Plasmodium falciparum NF54 DNA was extracted from FTA cards and *An. stephensi* mosquitoes using the Qiagen QIAamp Mini DNA extraction kit according to the manufacturer's protocol for tissue samples with the additional step of a 15 min incubation of the FTA card in tissue lysis buffer ATL (Qiagen, Valencia, California). All DNA was eluted in 50 µl of molecular biology grade water and then stored at -80°C or used immediately.

Real-time PCR (qPCR) detection

Reverse transcriptase (RT) qPCR was run in duplicate for each RNA extract using an ABI7500 Fast platform (Life Technologies, Grand Island, New York) and a set of Taqman primers/probe designed to detect all four DENV serotypes targeting conserved nucleotides in the 3'UTR¹⁵. The SuperScript III Platinum One-step RT-qPCR kit (Life Technologies, Grand Island, New York) was used for all RT-qPCR reactions. Each reaction contained 250 nM forward primer (5'-GARAGACCAGAGATCCTGCTGTCT-3'), 500 nM reverse primer (5'-ACCATTCCATTTCTGGCGTT-3'), 100 nM probe (5'-AGCATCATTCCAGGCAC-3') labeled with 5' FAM and a minor groove binder and a non-fluorescent quencher on the 3' end, 50 nM ROX passive reference dye, and 7 µl of FTA card extract for a total reaction volume of 20 µl. The cycling conditions included a reverse transcription pre-incubation at 40°C for 30 min, followed by enzyme activation at 92°C for 2 min, and 45 cycles of 92°C for 1 sec and 60°C for 30 sec. Positive samples had a cycle threshold (Ct) value ≤ 39.9. Samples with Ct values >39.9 were considered on a case-by-case basis.

The first qPCR assay employed as described by Bass *et al*¹⁶ (Bass assay) is *Plasmodium* species-specific with primers and probes targeting a region in the 18S rRNA gene containing two single nucleotide polymorphisms

that are highly specific to *P. falciparum*. This assay was originally developed to differentiate between the four different human species of *Plasmodium*. The qPCR reaction (20 µl) comprised of 4 µl DNA template, 10 µl of 2×SensiFAST™ (Bioline, Taunton, Massachusetts), 800 nM forward PlasF primer (5'-GCTTAGTTACGATTAATAGGAGTAGCTTG-3'), 800 nM reverse primer PlasR (5'-GAAAATCTAAGAATTTACCTCTGACA-3'), and 300 nM Falcip+ probe (5'-TCTGAATACGAATGTC-3') labeled with 6-FAM at the 5' end. PCR grade water was added to bring the reaction to its final volume. The reaction was run on an ABI7500 Fast platform with the following thermal profile: 10 min at 95°C, followed by 40 cycles of 95°C for 10 sec and 60°C for 45 sec. Samples considered positive had a cycle threshold (Ct) value ≤ 39.9. Samples with Ct values > 39.9 were considered on a case-by-case basis.

The second qPCR assay employed as described by Kamau *et al*¹⁷ (Kamau assay) is a genus-specific assay that also targets the 18S rRNA gene, since it is highly conserved among *Plasmodium* spp. This assay was run in comparison to the Bass assay to examine how a sensitivity difference between assays may affect pathogen detection. The qPCR reaction (5 µl) comprised of 1 µl DNA template, 2 µl of 2 × QuantiTect™ probe master mix (Invitrogen, Waltham, Massachusetts), 0.05 µM of QuantiTect™ RT mix, 0.4 µM forward primer Plu3F (5'-GCTCTTTCTTGATTTCTTGGATG-3'), 0.4 µM reverse primer Plu3R (5'-AGCAGGTTAAGATCTCGTTCG-3') and 0.2 µM probe Plu3 (5'-ATGGCCGTTTTTAGTTTCGTC-3') labeled with Cy5 at the 5' end (Integrated DNA Technologies, Coralville, Iowa). PCR grade water was added to bring the reaction to its final volume. The reaction was run on an ABI7500 Fast platform with the following cycling conditions: 30 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60°C for 1 min. Samples considered positive had a cycle threshold (Ct) value ≤ 39.9. Samples with Ct values > 39.9 were considered on a case-by-case basis.

Pathogen detection on and off FTA cards

Standard curves were generated with and without the presence of FTA cards using DENV-2 viral stock, strain New Guinea C, diluted 1:10 in molecular biology grade water from 1000 PFU/µl to 0.1 PFU/µl. One µl of each diluted virus standard was added to honey-soaked 1cm² Clonesaver FTA® cards in triplicate and allowed to dry at room temperature for 1 h or added in triplicate directly into lysis buffer (Qiagen, Valencia, California).

Salivary glands from female *An. stephensi* mosquitoes (12 days post-*P. falciparum* NF54 strain infection)

were dissected and spun down through glass wool to release sporozoites into a 1× PBS + 1% BSA solution. Sporozoites were counted using a haemocytometer and five-fold dilutions ranging from 2533.3 sporozoites/μl to 0.03 sporozoites/μl were made to prepare a standard curve with both the Bass and Kamau assays. Ten μl of each diluted sporozoite standard was added to honey-coated 1 cm² Clonesaver FTA[®] cards in triplicate and allowed to dry at room temperature for 1.5 h (Whatman, Piscataway, New Jersey) or added in triplicate directly into lysis buffer (Qiagen, Valencia, California).

Statistical analysis

Comparisons of replicate Ct values of DENV-2 and *P. falciparum* NF54 were made with multiple *t*-tests where significant differences were noted with $p \leq 0.05$. All statistical tests were performed on GraphPad Prism[®] version 6.04 for Windows, GraphPad Software (La Jolla, California).

RESULTS

Mosquito survival and FTA card storage tests

For best longevity and vigour, female mosquitoes were housed with a water source to ensure 100% survival rates up to 72 h (data not shown) as well as efficient sugar feeding in the experimental setup (Fig. 1a). Additionally, the three different FTA card storage conditions did not reveal any statistically significant results of pathogen detection when the Ct values of qPCR were compared (data not shown). Hence, it was decided to place all collected experimental FTA cards into microcentrifuge tubes containing desiccant beads at 4°C overnight before processing.

Dengue viral RNA detection on FTA cards

Prior to conducting sugar-feeding experiments with mosquitoes, it was imperative to examine whether or not DENV-2 RNA could be detected on FTA cards. Thus, DENV-2 standard curves were generated using specific PFU amounts of stock virus both with and without the Clonesaver FTA[®] cards. Dilution points for DENV-2 (five points from 0.1 to 1000 PFU/μl) were tested with FTA cards and the same five were tested without the cards. Virus was detected from samples (with and without FTA cards) at all five dilution points. Extraction from the FTA cards resulted in an overall increase in Ct value as well as a slight increase in the deviation between replicates (Fig. 2). Therefore, DENV-2 can be detected on FTA cards, but a log of detection sensitivity is lost. Honey alone or honey and dye addition to the FTA cards was not

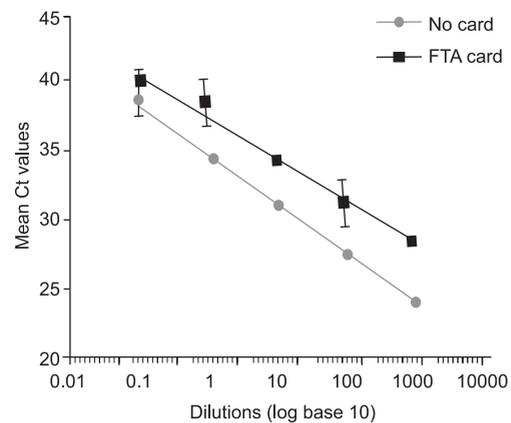


Fig. 2: Standard curves of DENV-2 particle detection off (no card) and on FTA cards. Linear regression was performed to model the relationship between total PFU and Ct value for both data sets. Mean and standard deviations are plotted.

found to confound extraction or inhibit RT-qPCR (data not shown).

Detection of dengue viral RNA from infected *Aedes aegypti* sugar-feeding on FTA cards

All *Ae. aegypti* mosquitoes from both sets survived the duration of the 6 h sugar feed. After sugar-feeding using the honey-soaked, dye impregnated Clonesaver FTA[®] cards, each *Ae. aegypti* mosquito was dissected to visualize ingested dyed honey in the gut and crop (Fig. 1b) and processed to detect infectivity with DENV-2. In both the sets, 100% of the mosquitoes tested were positive for DENV-2 (Table 1a). Next, each of the associated FTA cards was processed for DENV-2 detection. In both the sets about 65% (average) of FTA cards tested were found positive for DENV-2 (Table 1a). As expected, the cards exhibited a significantly higher Ct value, approximately 36.0 as compared to the average 20.0 for the infected mosquitoes. It should be noted that two additional uninfected mosquitoes were used for each experimental set, and they as well as their associated cards were confirmed negative for DENV-2 (data not shown).

To further determine why approximately a third of the associated cards remained negative for DENV-2, the feeding behaviour of each mosquito was explored. It is reasonable to assume that if a mosquito did not feed then its associated card should be negative. In both the sets of 30 mosquitoes, dissection revealed similar ingestion rates with 29/30 (97%) mosquitoes feeding in Set 1 and 27/30 (90%) mosquitoes in Set 2 (Table 1b). For Set 1, the one mosquito that did not feed had a negative card. For Set 2, two of the three mosquitoes that did not feed had negative cards; however, one mosquito that did not feed turned up with a positive card suggesting that sa-

Table 1a. DENV-2 infected mosquito sugar-feeding FTA card experiments (n = 30)

	Set 1 (Day 12, 6 h)		Set 2 (Day 14, 6 h)	
	Inf Mosq	FTA card	Inf Mosq	FTA card
Positive	30/30 (100)	20/30 (67)	30/30 (100)	19/30 (63)
Negative	0/0 (0)	20/30 (33)	0/0 (0)	11/30 (37)
Ct value	18.3 ± 1	35.4 ± 2.7	22.5 ± 1.6	36.7 ± 2.2

Inf Mosq—Infected mosquitoes; Figures in parentheses indicate percentages.

Table 1b. Further breakdown of Table 1a to include the conditions of whether or not a mosquito actually fed (n = 30)

Condition	Set 1	Set 2
	(Day 12, 6 h)	(Day 14, 6 h)
Fed	29/30 (97)	27/30 (90)
Did not feed	1/30 (3)	3/30 (10)
Fed and FTA card positive	20/29 (69)	18/27 (67)
Fed and FTA card negative	9/29 (31)	9/27 (32)
Did not feed and FTA card positive	0/0 (0)	1/3 (33)
Did not feed and FTA card negative	1/1 (100)	2/3 (67)

Figures in parentheses indicate percentages.

liva was deposited without the honey/dye being ingested (probing) (Table 1b).

The number of dengue PFUs deposited on FTA cards during sugar-feeding

Using the DENV-2 standard curves developed with and without FTA cards (Fig. 2), the approximate number of PFU deposited on cards through the sugar-feeding experiments was determined (Fig. 3). The box and whisker plots for both experimental feeding sets illustrate that the median number of PFUs deposited was approximately 1, although the range was from 0 to >50, as determined by the standard curve generated from the FTA cards. This means that a single DENV PFU deposited on a FTA card

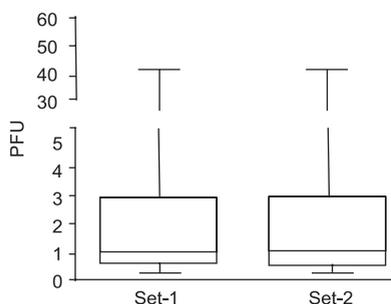


Fig. 3: Approximate number of DENV plaque forming units (PFU) deposited on FTA cards. Set 1 represents DENV-2 infected mosquitoes (Day 12, 6 h) that fed and had positive associated FTA cards. Set 2 represents DENV-2 infected mosquitoes (Day 14, 6 h) that fed and had positive associated FTA cards. Box and whiskers plot depicts quantity of DENV reported in PFU, including fractional quantities. Horizontal bars show median, first and third quartiles for each set. Whiskers represent outliers.

by a mosquito can be detected, which confirms that the standard curves produced were successful predictors of these detection limits.

Plasmodium falciparum sporozoite DNA detection on FTA cards

Since *P. falciparum* is a parasite and more complex than an RNA virus such as dengue, two different malaria qPCR assays were investigated with respect to potential detection on FTA cards, one genus-specific (Kamau assay) and one species specific (Bass assay). When each assay was used to detect *P. falciparum* sporozoites directly from lysis buffer, the Bass assay proved to be more specific and sensitive than the Kamau assay, as it was able to detect less than a full sporozoite and showed lower Ct values for each dilution point (Fig. 4).

As with dengue, it was important to determine whether or not *P. falciparum* DNA could be detected on FTA cards prior to conducting sugar-feeding experiments with mosquitoes. Thus, *P. falciparum* standard curves were generated using sporozoites spiked directly into lysis buffer solution (without FTA cards) and from sporozoite DNA extracted off of Clonesaver FTA® cards. For each, with and without card scenario, the Kamau and Bass qPCR assays were compared to determine the detection power on FTA cards. For the Kamau assay, the limit of detection was found to be 6 sporozoites when processing DNA directly from lysis buffer. However, DNA processed off of FTA cards had a limit of detection at 60 sporozoites (Fig. 5a). For the Bass assay, the limit of detection was approximately one sporozoite when DNA was processed off of FTA cards (Fig. 5b). Since both assays exhibited detection at 60 sporozoites, it was extrapolated so that as few as 60 sporozoites deposited on FTA cards by mosquitoes while sugar-feeding could be detected by either assay. Extraction from the FTA cards resulted in an over-

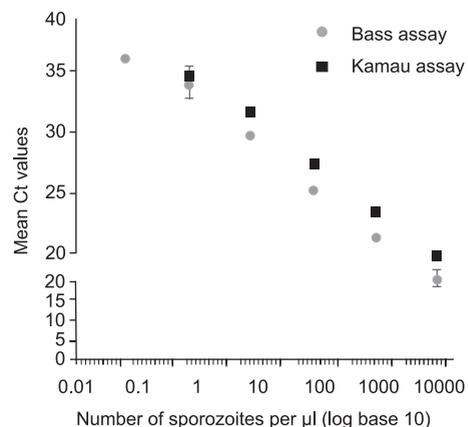


Fig. 4: Assay comparison for detection of *P. falciparum* sporozoites directly from lysis buffer (without FTA card).

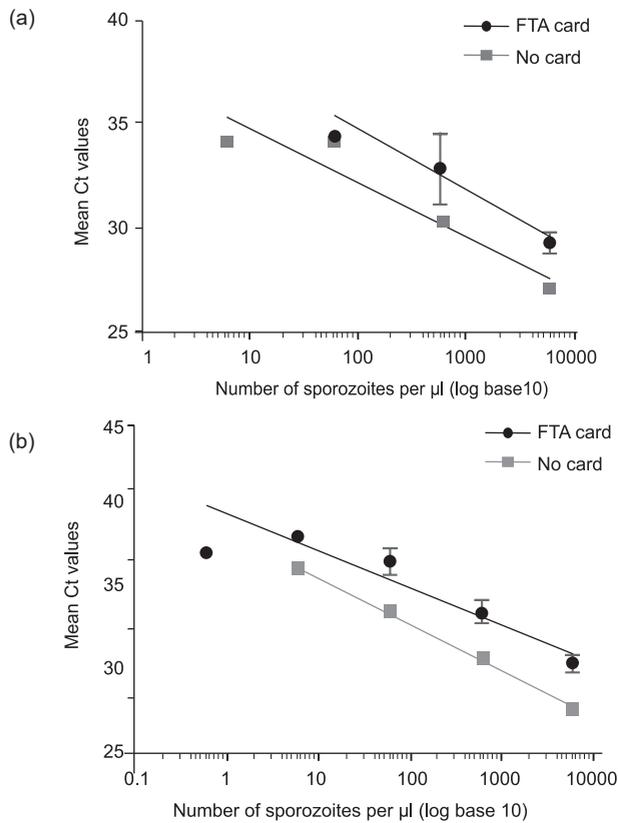


Fig. 5: Standard curves of *P. falciparum* detection off (no card) and on FTA cards. Linear regression was performed to model the relationship between sporozoite number and Ct value for both data sets. Mean and standard deviation are plotted above. Only samples positive for sporozoite DNA were plotted. Detection was performed using (a) Kamau assay; and (b) Bass assay.

all increase in Ct value as well as a slight increase in the deviation between replicates with a loss of about a log in detection sensitivity (Figs. 5a and b). The same phenomenon was observed with the dengue samples. Also, honey alone or honey and dye addition to the FTA cards was not found to confound extraction or inhibit qPCR (data not shown).

Detection of Plasmodium falciparum sporozoite DNA from infected Anopheles stephensi sugar-feeding on FTA cards

With regard to *P. falciparum*, the *An. stephensi* exhibited higher rates of sugar-feeding at the 24 h (37%) and 65 h (45%) time points as compared to the shorter

6 h time point, that was successful with dengue and *Ae. aegypti* (data not shown). Since the feeding was highest at the 65 h time point, all 20 mosquitoes from this set were examined *via* qPCR following both the Kamau and the Bass assays, showing 45% (9/20) and 65% (13/20) of the associated FTA cards were positive, respectively (Table 2). The variation in the percent positive cards is indicative of the limits of the various assays used to detect the sporozoites. Of note is mosquito number 17, which showed an opposite result of what was expected. Upon further examination of the data for number 17, both Ct values for the Kamau and Bass assays were close to the positive call Ct cut-off value of 39.9, with the Bass assay ultimately being considered negative.

Plasmodium falciparum sporozoites can be detected with both assays on FTA cards after 65 h of mosquito sugar-feedings. However, since the Bass assay exhibited superior detection, the remaining two sets of *An. stephensi* (6 and 24 h time points) FTA cards were only tested with the Bass assay. The results from all the three sets of mosquitoes are shown in Table 3. It should be noted

Table 2. Assay comparison of FTA card results from the 65 h time point of *P. falciparum*-infected mosquitoes sugar-feeding

Mosquito number	FTA card PCR result	
	Kamau assay	Bass assay
1.	Positive	Positive
2.	Negative	Negative
3.	Negative	Positive
4.	Negative	Negative
5.	Negative	Negative
6.	Negative	Negative
7.	Positive	Positive
8.	Positive	Positive
9.	Positive	Positive
10.	Negative	Positive
11.	Positive	Positive
12.	Negative	Positive
13.	Positive	Positive
14.	Negative	Negative
15.	Negative	Negative
16.	Negative	Positive
17.	Positive	Negative
18.	Negative	Positive
19.	Positive	Positive
20.	Positive	Positive

Highlighted rows signify where the Kamau and Bass assays produced different results.

Table 3. Bass assay results from three different *P. falciparum*-infected mosquito sets

	Set 1 (Day 12, 6 h) n=20		Set 2 (Day 12, 24 h) n= 20		Set 3 (Day 12, 65 h) n = 20	
	Inf mosq	FTA card	Inf mosq	FTA card	Inf mosq	FTA card
Positive	20/20 (100)	2/20 (10)	20/20 (100)	7/20 (35)	20/20 (100)	13/20 (65)
Negative	0/0 (0)	18/20 (90)	0/0 (0)	13/20 (65)	0/0 (0)	7/20 (35)
Ct Value	25.4 ± 1.9	33.2 ± 2.2	23.5 ± 1.3	33.8 ± 2.3	22.5 ± 1.1	31.7 ± 2.7

Inf mosq—Infected mosquitoes; Figures in parentheses indicate percentages.

that two additional uninfected mosquitoes were used for each experimental set, and they as well as their associated cards were confirmed negative for *P. falciparum* (data not shown).

DISCUSSION

Some of the earliest experiments to determine the infectivity of mosquitoes by exploiting sugar-feeding were performed in the early 1990s. At that time, nitrocellulose membranes (NCM) soaked in fructose were used to capture mosquito saliva, and a modified western blotting technique was used to detect the pathogen of interest. Results showed that pathogens could be identified from as few as 10 infected mosquitoes⁷⁻⁸. In this study, FTA cards were used in place of the NCM to better capture and preserve nucleic acids, and qPCR, a more sensitive and robust technique than western blotting, was used for pathogen detection. Using these materials and methods, DENV particles and malaria parasites were detected from a single mosquito sugar-feeding.

Although this study was successful in determining if a single mosquito is infected with a pathogen and capable of transmitting it, there are many reasons why this detection methodology is challenging. First, a number of variables affect mosquito sugar-feeding, such as behavioural, physiological, and structural specializations for finding, feeding on, and processing sugar, all of which were not accounted for in this study¹⁸⁻²⁰. Second, individual mosquito species have been shown to expectorate radically different numbers of malaria sporozoites when blood-feeding as compared to other species, and within the same mosquito species, they often expectorate very different numbers of sporozoites from bite-to-bite²¹. The same trend is seen with DENV, where mosquitoes infected by feeding on a higher viral titre blood meal expectorated more virus particles upon subsequent blood feeding once they became infectious²². It is unclear whether the same phenomenon is true for sugar feeding; however, it was assumed to be in this study. Third, saliva volume expectorated from mosquitoes varies among mosquito species and in *Ae. aegypti* declines with age, something that was not measured in this study²². Fourth, the quantities of malaria parasites and DENV particles expectorated were close to the limit of detection of the qPCR assays used, which can result in false negatives and lack of detection. Lastly, nucleic acid recovery from any collection strategy (in this case, FTA cards) is never 100% efficient and can vary from sample to sample making detection and accurate quantification difficult.

Standard curves are performed on known sample amounts to establish a baseline for comparison as well as

to establish limits of assay detection. For this study, the DENV standard curve was generated without any issue. However, the attempts at generating a sporozoite standard curve were very inconsistent. It was exceedingly difficult to generate consistent serial dilutions of sporozoites, which may have been due to their tendency to aggregate and stick to tubes and pipette tips. To mitigate these issues, PBS +1% BSA was used to prevent aggregation and silicone coated pipette tips and tubes to prevent sticking. However, these proved less than corrective, so there were still inconsistencies in the data. For example, the data collected for Fig. 4 showed that the Bass assay can detect less than one whole sporozoite; however, the standard curve data for the Bass assay (Fig. 5b) is lacking the two highest dilutions (without the FTA card).

It is surmised that different genera of mosquitoes sugar-feed at various times even after being sugar-starved for the same periods of time. This was observed with the initial feeding studies on *P. falciparum* infected *An. stephensi*. Even after an allotted 6 h of sugar-feeding time where the mosquitoes were in close proximity with honey soaked cards as the only food source, there was little evidence of feeding. These results revealed that sugar-feeding times were longer for the *P. falciparum* infected *An. stephensi* (24 and 65 h). This same delayed feeding was not seen with DENV-2 infected *Ae. aegypti*, which reinforces genus-specific differences in sugar-feeding and suggests that host parasite interactions may also influence sugar-feeding. Indeed, there are some studies that indicate that infection and even the stage of infection with a pathogen can impact the blood-feeding and host-seeking behaviours of mosquitoes²⁰. However, little is known regarding how mosquito infection status may impact sugar-feeding behaviour.

Since malaria, dengue, and leishmaniasis are three of the top ranking diseases in the world, and this study was able to detect DENV particles and malaria parasites after a mosquito sugar-feed, an additional study to detect *Leishmania* parasites on FTA cards fed on by infected sandflies was attempted. However, no *Leishmania* positive saliva was detected, likely due to which tissues in the sandfly become infected, and the way in which sandflies sugar-feed. *Leishmania* parasites colonize the sandfly midgut and damage the valves responsible for holding it closed without disseminating to the salivary glands. Infected sandflies regurgitate during subsequent blood feeding thereby transferring the parasite to the host²³. Conversely, when sandflies sugar-feed, their saliva does not contain parasites and the sugar is directed to the crop with no opportunity for parasites to be regurgitated from the midgut²⁴. Alternate methods for detecting whether

Leishmania infected sandflies are transmitting their parasites need to be explored.

The technique of using mosquito saliva to detect pathogens has come a long way since the initial western blot experiments, but it still needs refinement. For example, with saliva capture on FTA cards, the stability of viral RNA on the cards is not definitive, and it is unknown how the addition of a sugar source to the cards will affect the long-term stability of nucleic acids. Additionally, there are the issues of getting positive associated FTA cards, when no apparent sugar-feeding from an infected mosquito has occurred (false positive); and negative associated FTA cards, when feeding from a known infected mosquito has occurred (false negative). Other studies have experienced these issues as well as getting positive detection results from associated cards in traps with only uninfected mosquitoes^{11, 13-14}. Some of the problems related to this issue have to do with sample preparation technique, potential contamination, insufficiently sensitive detection assays, and whether or not the mosquito is actually transmitting. Finally, although FTA cards recently have been successfully fielded in carbon dioxide-baited light traps on an encephalitis hunt in Australia, their efficacy and operational feasibility as a surveillance tool specifically for malaria and dengue has yet to be determined²⁵.

In the future, this sample collection method could be combined with a novel mosquito control technology in development—attractive targeted/toxic sugar baits (ATSB)²⁶. The attractiveness of the FTA cards could also be maximized with attractants superior to honey. This would allow the deployment of the cards independently of a traditional trap, further reducing the resources required for vector and pathogen surveillance (Fig. 6). Low-tech and affordable, this concept device would overcome affordability challenges with conventional traps, thus providing opportunities to expand the surveillance coverage while significantly alleviating the current sample collection bottle neck. This type of system could be used by public health elements to determine disease risk in an area of operation in a shorter amount of time with less risk to field researchers, complement current detection technologies such as qPCR and ELISA, and allow for a more precise estimation of risk to a population by identifying what pathogens are circulating and the separation of infected and infectious vectors.

CONCLUSION

Given the increasing number of vector-borne disease threats and their staggering global burden, improved methods to efficiently detect when and where people are at

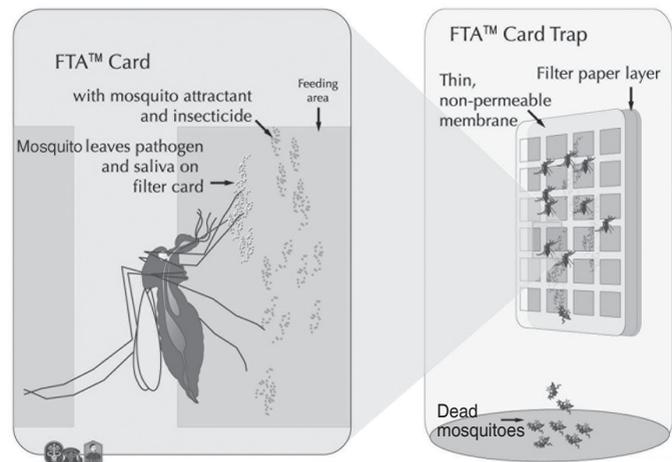


Fig. 6: Model of a non-traditional trap that incorporates FTA card concept. In the model, a mosquito attractant as well as an insecticide has been added to the FTA card. Therefore, when the mosquito feeds, it deposits saliva with any potential pathogens on the card while also ingesting the insecticide. The insecticide then kills the mosquito as shown (Source: Entomology Branch, the Walter Reed Army Institute of Research, Silver Spring, Maryland).

greatest risk are essential to support public health decision making. Vector-based surveillance programmes can provide vital information to save lives and prevent disease, but are plagued by low efficiency. New, innovative approaches to exploit mosquito sugar-feeding behaviour through the use of attractive, saliva capture platforms offer promise to overcome these limitations. There is growing evidence that a variety of pathogens can be detected in mosquito saliva samples following sugar-feeding. This study showed that DENV particles and malaria parasites transmitted by mosquitoes can be detected through a single sugar-feeding event using FTA cards. This infective mosquito sampling technique could be leveraged and adapted to exponentially expand vector-pathogen detection capacity or combined with an ATSB control approach to both surveil and control vector populations more efficiently.

ACKNOWLEDGEMENTS

The authors would like to thank Jessica Scheirer, Maj. Silas Davidson, and David Markman for their support and technical assistance and Judith Stoeffler for the graphic design. Special thanks are due to the funders: Military Infectious Diseases Research Program (U0381_14_WR_PP), Armed Forces Health Surveillance Branch (formerly known as GEIS – P0147_14_WR), and Medical Countermeasures Systems (formally known as CBMS-Plan # 15257).

This material has been reviewed by the Walter Reed Army Institute of Research, Entomology Branch. There

is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defence.

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Received: 22 May 2017

Accepted in revised form: 22 September 2017